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13. ABSTRACT *(Maximum 200*

The purpose of this project is to provide a rationale and pre-clinical evaluation of p53-based approaches to growth suppression and therapy sensitization of breast cancer. The scope of the first year's study was to evaluate drugs that could be used in combination with p53 to achieve enhanced tumor suppression, and to develop in vitro DNA repair assays that would enable us to study how p53-mediated suppression was affected by DNA repair. We have observed that p53 sensitizes breast cancer cells to DNA damaging chemotherapeutic drugs such as cisplatin, and to agents that inhibit DNA repair, but not to Taxotere™, a mitotic inhibitor, or 5-fluorouracil, an antimetabolite. Using DNA repair assays we have observed a correlation between down regulation of DNA repair, and increased sensitivity to p53. These results suggest a new biological strategy for breast cancer treatment employing p53 in combination with DNA repair inhibitors or with DNA damaging agents.

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Lucia A Gray 7.24.97

PI - Signature Date

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Growth Suppression and Therapy Sensitization of Breast Cancer
DAMD17-96-1-6038
Progress Report #1

INTRODUCTION

This project focuses on the development of p53-based approaches to growth suppression and therapy sensitization of breast cancer. p53 abnormalities occur in about 40% of breast cancers (1-4), and are a feature of more aggressive tumors showing increased aneuploidy and genetic instability (5,6). Because p53 gene transfer appears to have minimal consequences for normal cells, p53 based approaches to cancer therapy hold promise as effective biological therapies with specificity for tumor cells.

The strong selection pressure to lose p53 in such a high percentage of breast cancers derives most likely from its role as a modulator of apoptosis following DNA damage (7-11). Karyotypic instability is inherent in cancer and generates strand breaks and other forms of DNA damage that can trigger p53-mediated apoptosis. Loss of p53 function would confer a growth advantage to these cells by removing the trigger for apoptosis that would normally eliminate them (12). In addition to promoting tumor progression, loss of p53-mediated apoptosis also confers resistance to DNA damaging chemotherapeutic drugs and radiation. This has been shown in studies using p53 null murine fibroblasts and hematopoietic cells (7,8,13), all of which were observed to be highly resistant to apoptosis following exposure to a variety of chemotherapeutic drugs and radiation. In our own studies, we have extended these results to tumor cells expressing mutant forms of p53. We have shown that these cells become markedly more sensitive to DNA damaging drugs and radiation following gene transfer of wild-type p53 (14).

The central hypothesis of this project is that the level of DNA damage constitutes a key determinant of a tumor cell's susceptibility to p53-mediated apoptosis. Tumor cells that fail to repair DNA damage, arising either from intrinsic genomic instability or from external DNA damaging agents might be expected to be particularly susceptible to p53 gene transfer. As we show below, several agents, acting through different mechanisms to induce DNA damage or to inhibit DNA repair, appear to work synergistically with p53 to enhance cell killing. These observations provide a rationale for a new biological strategies for the treatment of breast cancer involving combinations of p53 with DNA damaging chemotherapeutic drugs or radiation, or with inhibitors of DNA repair.

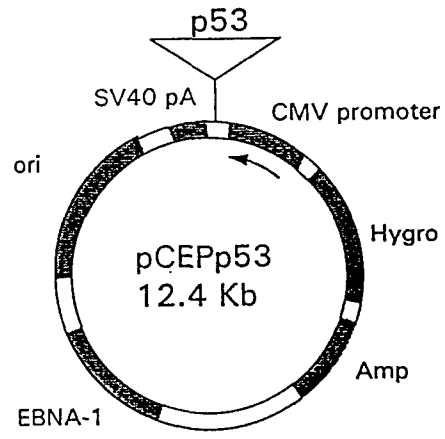
BODY OF REPORT

Our objectives for this first year, as outlined in the Statement of Work included (Task 1) *in vitro* drug sensitivity assays in order to identify drugs that worked best in combination with p53, and (Task 2) development of DNA damage assays in order to measure DNA repair and study how p53-mediated suppression was affected by DNA repair.

Results

Selection of modified clones of T47D cells expressing wild-type p53. For *in vitro* drug sensitivity assays, clones of wild-type-p53-expressing T47D cells were selected in 50 μg/ml hygromycin following transfection of the cells with the pCEP4p53 plasmid (shown below) as described in Methods. This plasmid does not integrate, but maintains sustained expression of the transgene as a result of its independent origin of replication. In this case, p53 is expressed from the CMV promoter. Following selection, expression of wild-type p53 was verified by a functional assay that measures transcriptional transactivation by p53.

We used the PG13 CAT reporter plasmid (provided by Dr. Bert Vogelstein, Johns Hopkins University) in which the bacterial chloramphenicol acetyltransferase (CAT) gene is placed downstream of a wild-type p53 binding site. 24 hours following transfection, cells expressing wild-type p53 will accumulate significant levels of CAT activity over background. Figure 1 shows the results of such an assay. T47Dp53 clone 9 was chosen for further assays as it consistently showed elevated levels of wild-type p53 expression. T47 D cells transfected with the unmodified pCEP4 vector were used as a background control.



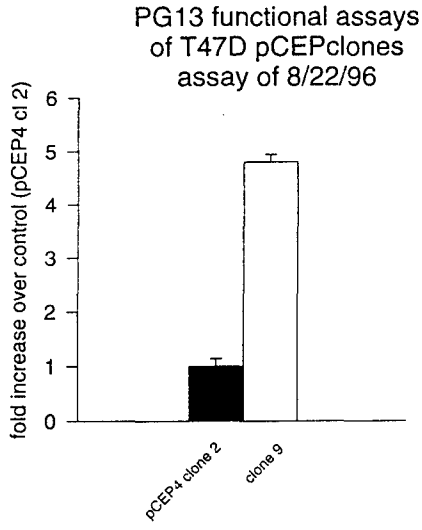


Figure 1. PG13CAT functional assay for wild-type p53. T47D clones modified with pCEP4 or with pCEP4p53 and selected in 50 μg/ml hygromycin were transfected with the PG13CAT reporter plasmid. Lysates prepared 24 hours post transfection were assayed for CAT activity.

In vitro drug sensitivity assays of T47D clones. Our preliminary studies showed that T47D cells transduced with wild-type p53 were more sensitive than control cells to the DNA damaging agent, cisplatin, consistent with our published results in other tumor types (14). In order to extend these observations to other classes of drugs, we tested the relative sensitivities of T47Dp53 clone 9 cells and T47DpCEP4 clone 2 cells (control) to cisplatin, 5-fluorouracil, and Taxotere.™ Cisplatin is a DNA damaging drug that reacts directly with the DNA to form primarily bifunctional adducts on adjacent guanine residues. 5-fluorouracil is an antimetabolite that inhibits thymidylate synthetase. Taxotere™ is a plant alkaloid that blocks microtubule

depolymerization and inhibits mitosis. As shown in Figure 2, wild-type p53-expressing T47Dp53 clone 9 cells were more sensitive to cisplatin, showed virtually no increased sensitvity to 5-fluorouracil, and were more resistant to Taxotere™. This suggests that expression of wild-type p53 may sensitize breast cancer cells to DNA damaging treatments, but not to treatments that do not directly damage DNA. Furthermore, drugs acting via a mitotic block such as Taxotere™ may be more effective in cells lacking p53.

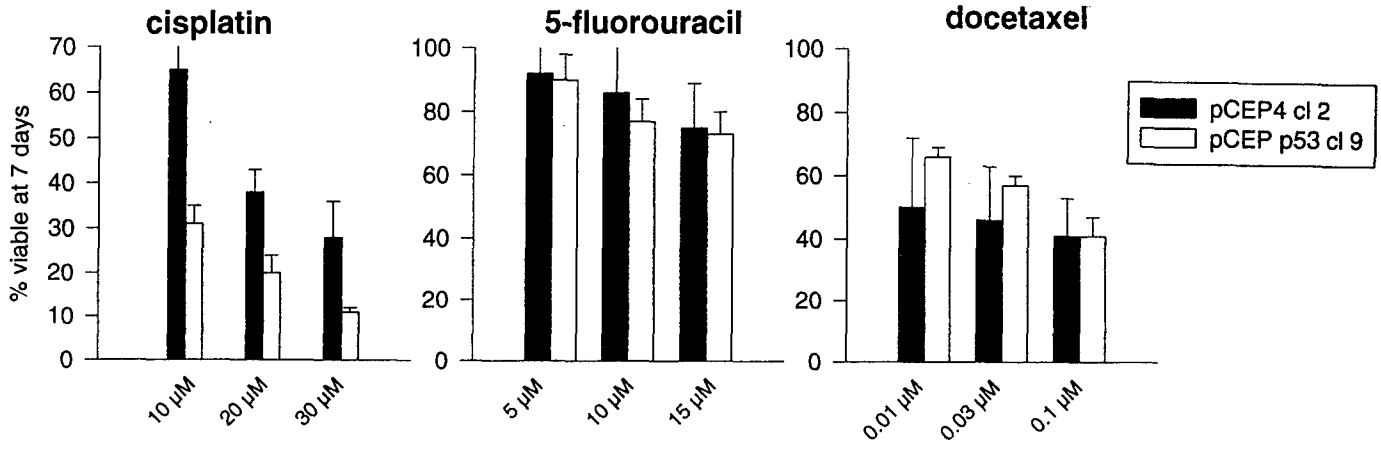
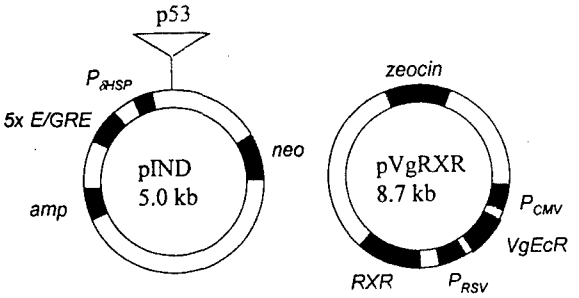


Figure 2. Sensitivity of T47DpCEP4 clone 2 (control) and T47DpCEP4p53 to cisplatin, 5-fluorouracil, and docetaxel (Taxotere™). Sensitivity was determined by a 96-well viability assay described in Materials and Methods.

Selection of T47D clones expressing an inducible p53. Several of the T47D clones selected above for constitutive expression of p53 appeared to lose p53 expression over time. This is not surprising given the selective pressure to lose negative growth regulators. Because this makes it difficult to maintain long term cultures of wild-type p53-expressing clones, we were interested in the new ecdysone-inducible mammalian expression system (15) that has become commercially available this year (Invitrogen). This system appears to offer significant advantages over previous inducible systems, including the system we initially proposed to use for *in vivo* studies in year 3 (zinc-inducible metallothionein promoter). The ecdysone-inducible system offers extremely low basal expression (which facilitates *in vitro* selection) and high inducibility for both animal studies and *in vitro* cell culture studies. Furthermore, unlike inducible systems based on eukaryotic promoters, there are virtually no secondary effects in mammalian cells from the insect hormone ecdysone. Thus the behavior of modified tumor cells exposed to ecdysone either in animals or in cell culture can be attributed entirely to the induced expression of p53.

The two plasmid vector system is shown below. Wild-type p53 was amplified by PCR from cDNA obtained from normal human skin fibroblasts in such a way as to create HindIII and EcoR1 sites at the 5' and 3' ends respectively. This fragment was gel purified and ligated into the multicloning site of pIND between the HindIII and EcoR1 sites to create pINDp53. Following subcloning and purification, pINDp53 in combination with pVgRXR (providing the nuclear receptor necessary for ecdysone-induced expression of p53.) and the p53-responsive PG13CAT reporter plasmid were co-transduced into p53 negative SaOS-2 cells to verify inducible expression of p53. Two days post-transduction cell lysates were prepared and assayed for

chloramphenicol acetyltransferase (CAT) activity. As shown in Figure 3, p53-mediated CAT expression was induced only in cells modified with pINDp53 and treated with the ecdysone analogue, muristerone.



two plasmid-ecdysone-inducible expression system.

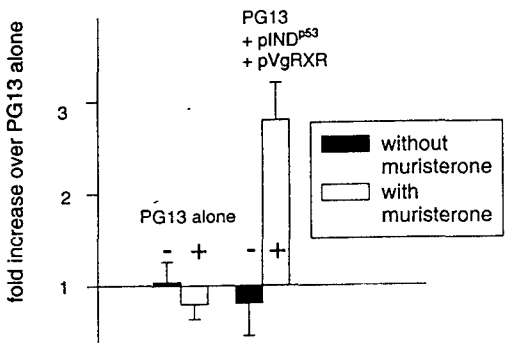


Figure 3. CAT assay of p53-negative Saos-2 cells following transfection with the PG13CAT reporter plasmid + pINDp53 + pVgRXR and treated sunsequently with (or without) the ecdysone analogue muristerone.

DNA damage assays based on PCR (PCR-stop assay). In order to study DNA repair, we set up a DNA damage assay known as the "PCR stop assay" (16-18): The assay is based on the principle that every DNA lesion, including platinum adducts produced by cisplatin, can potentially block the progression of the Taq polymerase and decrease the yield of a given PCR product. It has been well demonstrated that the degree of inhibition of PCR correlates with the level of platination, indicating that the polymerase is inhibited by every lesion (18). In addition, when whole cells are incubated with varying levels of cisplatin, the degree of inhibition of amplification of a specific PCR product from DNA purified from these cells, correlates closely with the amount of DNA damage (level of DNA platination) as measured by atomic absorption (18). We have synthesized the following primers used by Oshita and Saijo (16), which amplify a 2.7 Kb fragment of the human hypoxanthine phosphoribosyl transferase (HPRT) gene:

5' primer: 5'-TGGGATTACACGTGTGAACCAACC-3'
3' primer: 5'-GATCCACAGTCTGCCTGAGTCACT-3'

As an internal control for the efficiency of the PCR reaction, we use a nested 5' primer which amplifies a 150 base fragment of the same gene. At levels of cisplatin used to treat cells, damage to the smaller fragment is undetectable.

nested 5' primer: 5'- CCTAGAAAGCACATGGAGAGCTAG-3'

Genomic DNA was prepared from about 10^6 cells using the Qiagen Qiamp Blood Kit™ following the manufacturer's instructions, and resuspended in sterile H_2O at a concentration of 0.5 mg/ml. PCR reactions are performed in 25 μl containing 50 mM KCL, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 250 μM each of dNTP, 1 μM of the forward and reverse primers and 0.1 μM of the nested primer, 0.25 μl Taq enzyme (Qiagen), 0.5 μl of Q™ solution (Qiagen), and 0.01 μM of ^{32}P-dATP and labeled probe. Quantitative amplification is as follows: one cycle (1'30" 94° C), 25 cycles (94°C for 1 minute, 57° C for 1 minute, 70° C for 2'30"), 1 cycle (94° C for 1 minute, 57°C for 1 minute, 70°C for 7 minutes, 4°C to hold). To confirm that the extent of reaction remains directly proportional to amount of template, we have performed control reactions with known amounts of DNA in 2 fold dilutions. For quantitation, The 3' primer which is common for the 2.7Kbp and 150 bp fragments, is end labeled with γ^{32}P-dATP and added at a concentration of 0.01 μM along with cold primers. Samples were analyzed on 1% agarose gels, enabling visualization of both the smaller and larger bands on the same gel. Figure 4 shows the process schematically, and shows a sample of results obtained with DNA from parental T98G cells treated with different doses of cisplatin for 1 hour.

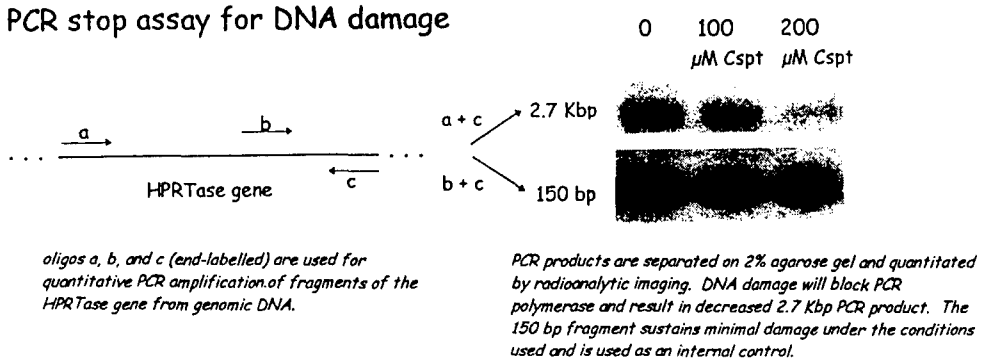


Figure 4. PCR stop assay showing primers used for amplification of a 2.7 Kb fragment and a 150 base fragment of the HPRTase gene from genomic DNA. of T98G cells treated with varying doses of cisplatin for 1 hour. Products are then analyzed on a 2% agarose gel in the presence of ethidium bromide as shown.

The gel is then partially dried for two hours to flatten it, and the bands quantitated using an Ambis4000 Radioanalytic Imaging System (Ambis, Inc., San Diego, CA). For each point, the intensity of the upper band (after correcting for the intensity of the lower band) is expressed as a percentage of the signal observed with DNA from untreated cells. Figure 5 shows the results after quantitation for T98G cells treated with 100μM or 200μM cisplatin. DNA damage is readily detectable at these doses and repair of damage can be quantitated by comparing DNA samples immediately after exposure to cisplatin, and 6 hours later. Also shown in the figure is an analysis of cells treated with cisplatin and incubated in the presence of 3-aminobenzamide, which is known to inhibit DNA repair through inhibition of ADP ribosylation (19). In DNA from T98G cells treated with 200 μM cisplatin, we observe a 50% - 70% drop in PCR signal strength. This corresponds to roughly 0.5-0.7 lesions per 2700 bases based on the formula # lesions= 1 - ($cpm^{damagrd}/cpm^{control}$), which applies under quantitative PCR conditions. By 6 hours, the signal strength for the cisplatin-treated T98G sample is 80-90% that of control cells, whereas the 6 hour sample from T98G cells treated

with cisplatin and 3-aminobenzamide appears unchanged. A parallel growth assay on these cells showed that 200 μM cisplatin reduced 7 day viability of parental cells to about 25-30% of untreated cells.

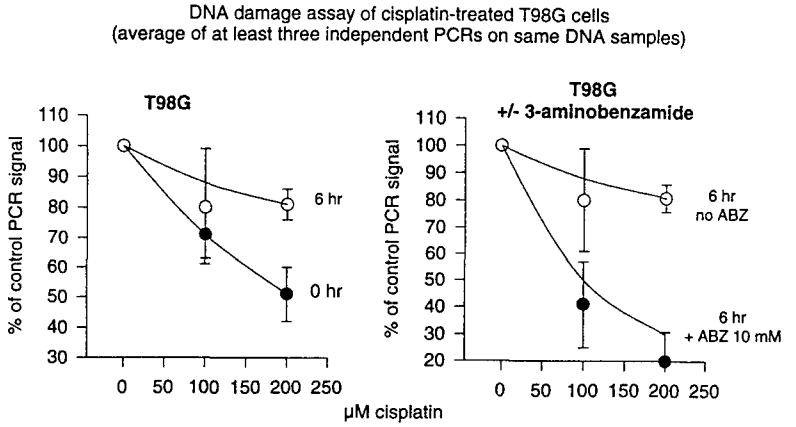


Figure 5. PCR stop assay of DNA from cisplatin treated cells, immediately after a 1 hour exposure to cisplatin, and 6 hours later. Left: T98G cells. Right: T98G cells incubated in the presence of 10 mM 3-aminobenzamide.

DNA repair assays based on host cell reactivation of a damaged plasmid. We have set up another convenient assay for measuring DNA repair based on the ability of a transfected cell to repair and express a cisplatin-damaged reporter plasmid encoding the chloramphenicol acetytransferase (CAT) gene. The plasmid we have used is pRcCAT, prepared by inserting the CAT gene coding sequence into the multicloning site of pRc/CMV (Invitrogen). Damaged plasmid was prepared by treating pRcCAT *in vitro* at a concentration of 0.33 mg/ml (in H_20) with 10 μM cisplatin (Platinol™, Bristol Laboratories) for 3 hours. The plasmid was then precipitated by the addition of ½ volume of 7.5M ammonium acetate and 1½ volumes of isopropanol at -20° C for 30 minutes. Plasmid was recovered by centrifugation, washed twice in 70% EtOH to remove traces of salt and resuspended in sterile H_2O for transfection. Under these conditions, expression of the damaged plasmid 24 hours following transfection drops to about 22% of the undamaged plasmid.

The assay was performed by plating 2 x 10^5 cells per well (in triplicate for each assay condition) in 24 well plates. The next day, cells were transfected as described above either with damaged or undamaged plasmid. As a control for basal levels of CAT activity in cell lysates, 3 wells of cells were left untransfected. One day following transfection or two days following transfection, triplicate wells were collected and cell lysates were prepared for measurement of CAT activity.

Role of DNA repair in p53-mediated suppression. DNA damage appears to provide a signal for p53-mediated apoptosis. Tumor cells able to repair the DNA damage generated by their intrinsic genomic instability might resist p53-mediated cell killing. We hypothesized that the success or failure of DNA repair could have significant bearing on the consequences of p53 expression in a karyotypically unstable tumor cell, and we wanted to test this directly.

In the model system shown in Figure 6a below, we used a rat tumor line modified to express wild-type p53 (9LpCEP4p53). The control line (9LpCEP4) was modified with the empty vector alone and expressed only endogenous mutant p53. Following plating in 96 well plates at 1000 cells per well, cells were treated with increasing concentrations of 3-aminobenzamide continuously for 7 days. 3-aminobenzamide inhibits the enzyme polyADP ribose polymerase, which is critical in DNA repair (ref 19, see also Figure 5). At 7 days cell viability was then determined (see methods). As shown in Figure 6a, cells expressing wild-type p53 were more sensitive to treatment with 3-aminobenzamide, supporting the hypothesis that down-regulation of DNA repair will enhance sensitivity to p53.

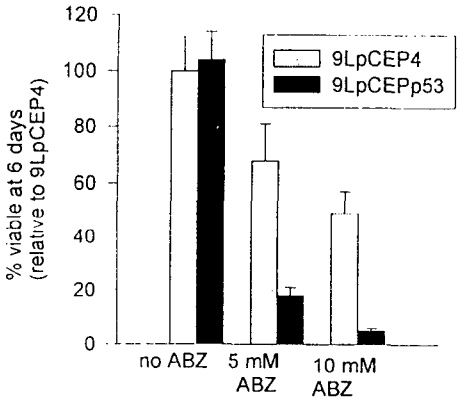


Figure 6a. Viability of 9LpCEP4p53 cells and 9LpCEP4 cells (control) 6 days following growth in 3-aminobenzamide.

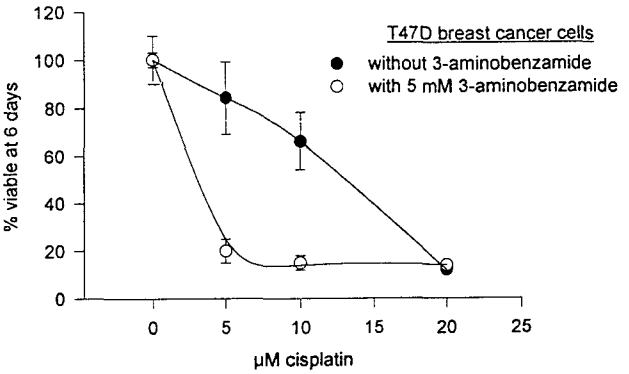


Figure 6b. Sensitivity of T47D breast cancer cells to cisplatin in the presence and absence of 3-aminobenzanide.

DNA repair may present a particular obstacle p53 therapy in late stage tumors that have been treated with therapy, where upregulation of DNA repair is a common mechanism of acquired therapy resistance (20-23). As shown in Figure 6b, repair appears to play and important role in the resistance of T47D breast cancer cells to cisplatin, as treatment of these cells with 3 aminobenzamide enhances their sensitivity to cisplatin in 96 well vaibility assays similar to those used in Figure 6a. Thus combination therapies using p53 along with inhibitors of DNA repair may constitute a new strategy for breast cancer therapy.

We have investigated combination approaches involving inhibition of the AP-1 transcription factor. A number of studies have implicated AP-1 in DNA repair and cisplatin resistance (20-24). AP-1 regulates the expression of a number of enzymes involved in DNA repair, including DNA polymerase ß, topoisomerase I, and dTMPsynthetase. Furthermore, phosphorylation of AP-1 via the jun kinase pathway accompanies exposure of cells to DNA damaging agents (25). Scanlon and coworkers ( ) have shown that down regulation of AP-1 through a ribozyme to c-fos (a component of the heterodimeric AP-1 complex) inhibits repair enzyme synthesis and reverses cisplatin resistance (24). Recently, we have extended these observations by showing that down-regulation of AP-1 phosphorylation inhibits DNA repair and reverses cisplatin resistance (reference 26, attached). We demonstrated this using cells modified with a dominant-negative

inhibitor of c-jun (mutant jun) which fails to be phosphorylated following DNA damage due to amino acid replacements at two critical phosphorylation sites. Cells modified to express mutant jun fail to repair cisplatin-induced DNA damage, as judged by the PCR stop assay (see Figure 7a and reference 26, attached), were markedly more sensitive to cisplatin (see reference 26, attached) and were more sensitive to suppression by p53 gene transfer (Figure 7b). This implicates an AP-1 regulated gene as a potential therapeutic target in combination with p53.

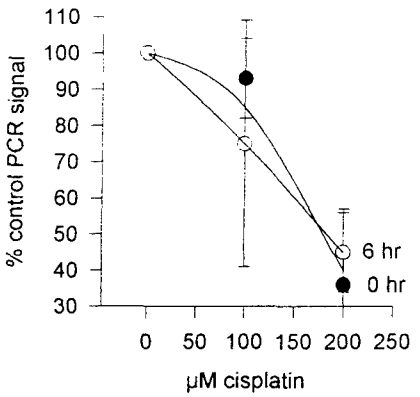


Figure 7a. PCR stop assay of DNA prepared from T98Gmutant jun cells. DNA was prepared either immediately after a one hour exposure to cisplatin or 6 hours later.

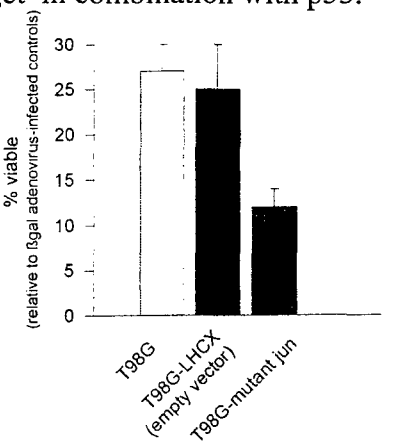


Figure 7b. Relative 7 day growth of p53-adenovirus-infected T98G subclones (compared to the same sunclones infected with control beta gal-infected adenovirus.

Because retinoids are known to display anti-AP-1 activity (27), we were interested in testing whether treatment of tumor cells would enhance suppression by p53 in the same manner that we had observed with mutant jun. As shown in Figure 8, T47D breast cancer cells grown in the presence of 9-cis retinoic acid do become more sensitive to suppression by p53. This result is consistent with our preliminary studies in which we observed enhanced sensitivity to p53 following treatment with the synthetic retinoid SR11220. We have used the plasmid reactivation assay to demonstrate that repair of a cisplatin damaged plasmid is inhibited by 9-cis retinoic acid in T47D breast cancer cells (Figure 9), and are presently using the PCR stop assay to confirm these results. This establishes at least one mechanism by which retinoic acid and its analogues could be acting to enhance p53-mediated suppression.

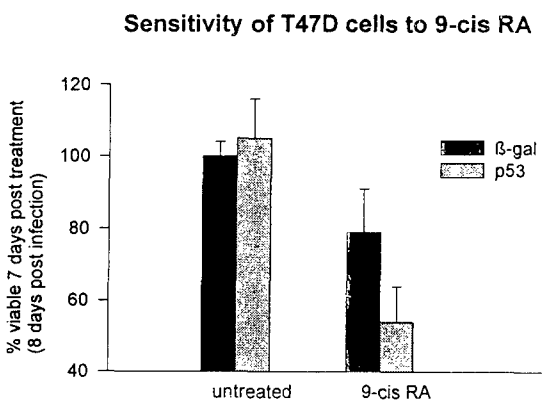


Figure 8. Viability of p53-adenovirus-infected T47D cells or ß-gal-adenovirus-infected T47D cells following 6 days of growth in the presence of 9-cis retinoic acid.

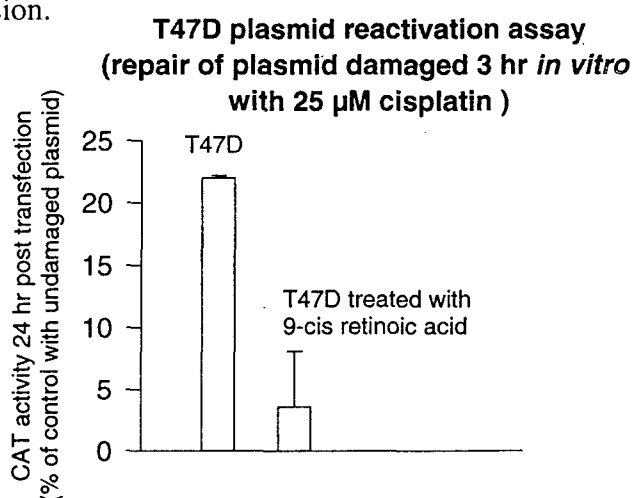


Figure 9. Plasmid reactivation assay demonstrating inhibition of repair of cisplatinum-damaged pRcCAT in T47D cells following treatmetn with 9-cis retinoic acid. Asays were performed 24 and 48 hours post-transfection with damaged pRcCAT, during which time cells received continous exposure to 1 μM 9-cis retinoic acid.

Conclusions

Our studies have shown that p53 is able to sensitize tumor cells to DNA damaging chemotherapeutic drugs such as cisplatin, and to drugs that inhibit DNA repair. We did not observe increased sensitivty of wild-type p53 modified breast cancer cells to agents that do not directly damage DNA. One drug, Taxotere™ (docetaxel), that acts by blocking the mitotic spindle, appeared to be more effective in cells expressing mutant p53. This suggests that drugs of this type could be highly efficacious in tumors expressing mutant p53.

In order to study the efficiency of DNA repair under various conditions, and to correlate DNA repair to p53-mediated apoptosis, we have set up two DNA repair assays, the PCR stop assay and the plasmid reactivation assay. These assays have enabled us to establish that down regulation of DNA repair correlates with enhanced sensitivity to p53, as we had originally proposed. We have down regulated DNA repair with several agents, including 3-aminobenzamide, an inhibitor of poly ADP ribosylation, 9-cis retinoic acid, and a dominant-negative inhibitor of c-jun phosphorylation. These observations are important because they suggest a new biological strategy for using p53 in combination approaches for breast cancer therapy.

We have prepared wild-type p53 expression vectors in which p53 expression is regulated by the insect hormone ecdysone. Subclones of T47D cells modified with these vectors will provide us with a tool to pursue further *in vitro* and *in vivo* studies of p53 combination therapies.

Materials and Methods.

Cell culture. The T47D breast cancer cells used in this work were purchased from ATCC and grown under 5% $C0_2$ in RPMI medium supplemented with 10% fetal calf serum, non-essential amino acids, glutamine, pyruvate and gentamycin. T47D clones modified with the pCEP4 vector or pCEPp53 were maintained under selection in 50 μg/ml hygromycin.

Plasmids. The plasmid pCEP4 was purchased from Invitrogen (San Diego, CA). When transduced into mammalian cells, this plasmid replicates as an independent cytoplasmic episome due to its EBV origin of replication. pCEP4 encodes the EBNA-1 protein needed for replication, and a hygromycin resistance marker. Transgene expression is driven by the CMV promoter. We have inserted the BamH1 fragment of the plasmid pLp53RNL (provided by Dr. Martin Haas, University of California San Diego), which contains the coding sequence of human wild-type p53, into the BamH1 site of pCEP4 to obtain pCEP4p53.

The reporter plasmid PG13 (PG13-CAT) was obtained from Dr. Bert Vogelstein (Johns Hopkins Oncology Center). In this plasmid, the chloramphenicol acetyl transferase (CAT) gene is under the control of a wild-type p53-specific binding site (28). Cells to be assayed for wild-type p53 expression were transfected (see below) with PG13 and incubated for two days at 37 oC in 5% CO2. Cell lysates were prepared and assayed for their ability to acetylate 14C-chloramphenicol as revealed by thin-layer chromatography of the reaction products (28). Alternatively, triplicate transfections with PG13 were performed in 24 well plates and lysates were assayed for their

ability to acetylate chloramphenicol using 3H-Acetyl CoA; in this case, the reaction products were extracted and quantitated by scintillation counting (29).

Transfections. For CAT assays and plasmid reactivation assays we have used a high efficiency transfection method based on cointernalization with an adenovirus capsid. Initially we used capsids to which polylysine tails had been chemically coupled. DNA complexed to the capsid through electrostatic interactions with the polylysine tails then entered the cell via the natural pathway of virus infection as described (30). This approach exploits the endosomolytic and nuclear localization properties of the virus capsid and results generally in higher levels of expression than other approaches. Furthermore, expression begins earlier after transfection than with other approaches, facilitating the plasmid reactivation studies where we want examine expression at early time points. The virus that we used was the replication-defective dl312 virus, purified over CsCl and stored in 10% glycerol in TE at pH 8. We now use a modified approach in which uncoupled virus, DNA, and polylysine are simply mixed together and incubated for one hour with cells (David Curiel, personal communication). We find that this approach, which relies on co-internalization of virus and DNA through fluid phase pinocytosis, is also very efficient, and avoids the need to chemically couple virus to polylysine. Specifically, 6 μg of DNA, 4 μg poly L-lysine, and 10^{10} viral particles in 1 ml of Hepes buffered saline pH8 are added for one hour to cells at 80% confluency in 6 cm plates (about 10^6 cells).

96-well viability assay. Cells are plated at 1000 cells per well in 96-well plates and treated the next day with various doses of drug. Quadruplicate wells are used for each condition. 5-6 days later (during which time control wells are in exponential growth) viability is measured by the MTT assay. This assay measures the bioconversion of a non-colored tetrazolium compound, MTT into the colored product, formazan. OD 490 nm is then measured using an ELISA reader.

References

1. Bartek, J., Iggo, R., Gannon, J., and Lane J.P. (1990) Genetic and immunochemical analysis of mutant p53 in human breast cancer lines. Oncogene, 5:893-899.
2. Coles, C., Condie, A.,.Chetty, U., Steel, C.M., Evans, A.J. and Prosser, J. (1992) Cancer Research 52:5291-5298.
3. Singh, S., Simon, M., Meybohm, I., Jantke, I., Jonat, W., Maass, H., and Goedde, H.W. (1993) Human breast cancer: frequent p53 allele loss and protein overexpression. Human Genet., 90:635-640.
4. Moll, U.M., Riou, G., and Levine, A.J. (1992) Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. Proc. Natl. Acad. Sci., USA 89:7262-7266.
5. Hendersin, I.C., Harris, J.R., Kinne, D.W., and Hellman, S. Cancer of the breast. *In* Cancer, Principles and Practice of Oncology. DeVita, V.T., Hellman, S., and Rosenberg, S.A., eds., J.P. Lippincott Comp., Philadelphia, 3rd ed., pp. 1197-1268.
6. Donehower, L.A., Godley, L.A., Aldaz, C.M., Pyle, R., Shi, Y-P., Pinkel, D., Gray, J., Bradley, A., Medina, D., Varmus, H.E. (1995) Deficiency of *p53* accelerates mammary tumorigenesis in *wnt-t* transgenic mice and promotes chromosomal instability. Genes and Development, 9: 882-895.

7. Lowe S.W., Ruley H.E., Jacks T., Housman, D.E. (1993) p53-mediated apoptosis modulates the cytotoxicity of anti-cancer agents. Cell 74: 957-967.
8. Lotem, J. and Sachs, L. (1993) Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents. Blood 82:1092-1096.
9. Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L., Wyllie, A.H. (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 362: 849-852.
10. Yonish-Rouach E, Resnitzky D, Lotem J, Sachs, L., Kimchi, A., Oren, M. (1991) Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. Nature 352: 345-347.
11. Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B., Costa, J. (1992) Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc Natl Acad Sci USA 89:4495-4499.
12. Lane, D. P.(1992) p53:guardian of the genome. Nature 358: 15-16.
13. Lowe, S.W., Bodis, S., McClatchey, A., Remington, L., Ruley, H.E., Fisher, D.E., Housman, D.E., Jacks, T. (1994) p53 status and the efficacy of cancer therapy *in vivo*. Science 266: 807-810.
14. Gjerset, R.A., Turla, S.T., Sobol, R.E., Scalise, J.J., Mercola, D., Collins, H., Hopkins, P.J. (1995) Use of wild-type p53 to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. Molecular Carcinogenesis 14: 275-285.
15. No, D., Yao, T.P., and Evans, RM. (1996) Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc Natl. Acad. Sci. USA 93: 3346-3351.
16. Oshita, F., Saijo, N. (1994) Rapid polymerase chain reaction assay to detect variation in the extent of gene-specific damage between cisplatin- or VP-16-resistant and sensitive lung cancer cell lines. Jpn. J. Cancer Res. 85: 669-673.
17. Oshita, F. and Eastman, A. (1993) Gene-specific damage produced by cisplatin, ormaplatin and UV light in human cells as assayed by the polymerase chain reaction. Oncology Research 5: 111-118.
18. Jennerwein, M.M. and Eastman, A. (1991) A polymerase chain reaction-based method to detect cisplatin adducts in specific genes. Nucleic Acids Research 19: 6209-6214.
19 Thraves, P., Mossmann, K.L., Brennan, T., Dritschilo, A. (1985) Radiosensitization of human fibroblasts by 3-aminobenzamide: an inhibitor of poly ADP ribosylation. Radiat. Res. 104: 119-127.
20. Scanlon, K.J., Kashini-Sabet, M., and Sowers, L.C. (1989) Overexpression of DNA replication and repair enzymes in cisplatin-resistant human colon carcinoma HCY8 cells and circumvention by azidothymidine. Cancer Comm. 1: 269-275.
21. Fravel, H.N.A., Rawlings, C.J., Roberts, J.J. (1978) Increased sensitivity of UV-repair-deficient human cells to DNA bound platinum adducts which, unlike thymidine dimers, are not recognized by an exonuclease from Micrococcus luteus. Mutat. Res. 51: 121-132.
22. Scanlon, K.J., Kashini-Sabet, M., Miyachi, H., Sowers, L., and Rossi, J. (1989) Molecular basis of cisplatin resistance in human carcinomas, model systems, and patients. Anti-cancer Res. 9:1301-1312.
23. Kashini-Sabet, M., Lu, Y., Leong, I., Haedicke, K., and Scanlon, K.J. (1990) Differential oncogene amplification in tumor cells from a patient treated with cisplatin and 5-fluorouracil. Eur. J. Cancer 26:383-390.

24. Scanlon, K.J., Jiao, L., Funato, T., Wang, W., Tone, T., Rossi, J.J., Kashini-Sabet, M. (1991) Ribozyme-mediated cleavage of c-fos mRNA reduces gene expression of DNA synthesis enzymes and metallothionein. Proc. Natl. Acad. Sci. USA 88: 10591-10595.
25. Adler, V., Fuch, S.Y., Kim, J., et al (1995) Jun-NH_2-terminal kinase activation mediated by UV-induced DNA lesions in melanoma and fibroblast cells. Cell Growth and Differentioation 6: 1437-1446.
26. Potapova, O., Haghighi, A., Bost, F., Liu, C., Birrer, M., Gjerset, R., Mercola, D. (1997) The jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. J. Biol. Chem. 272: 14041-14044.
27. Schule, R., Rangarajan, P., Yang, N., et al (1991) Retinoic acid is a negative regulator of AP-1 responsive genes. Proc Natl. Acad. Sci. USA 99: 6092-6096.
28. Kern SE, Pietenpol JA. Thiagalingam S, et al (1992) Oncogenic forms of p53 inhibit p53-regulated gene expression. Science 256: 827-830.
29. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: a Laboratory Manual, 2nd edition, C Nolan, ed. Cold Spring Harbor Press, 1989.
30. Curiel DT, Wagner E, Cotten M (1992) High efficiency gene transfer mediated by adenovirus coupled to DNA polylysine complexes. Human Gene Therapy 3: 147-154.

APPENDIX

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Communication

The Jun Kinase/Stress-activated Protein Kinase Pathway Functions to Regulate DNA Repair and Inhibition of the Pathway Sensitizes Tumor Cells to Cisplatin*

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We have studied the role of Jun/stress-activated protein kinase (JNK/SAPK) pathway in DNA repair and cisplatin resistance in T98G glioblastoma cells. JUN/SAPK is activated by DNA damage and phosphorylates serines 63 and 73 in the N-terminal domain of c-Jun, which is known to increase its transactivation properties. We show that treatment of T98G glioblastoma cells with cisplatin but not the transplatin isomer activates JNK/SAPK about 10-fold. T98G cells, which are highly resistent to cisplatin (IC_{50} = 140 ± 13 μM), modified to express a nonphosphorylatable dominant negative c-Jun (termed dnJun) exhibit decreased viability following treatment with cisplatin, but not transplatin, in proportion ($r_{Pearson}$ = 0.98) to the level of dnJun expressed leading to a 7-fold decreased IC_{50}. Similar effects are observed in U87 cells, PC-3 cells, and MCF-7 cells, as well as in T98G cells modified to express TAM-67, a known inhibitor of c-Jun function. In contrast, no sensitization effect was observed in cells modified to express wild-type c-Jun. Furthermore, through quantitative polymerase chain reaction-stop assays, we show that dnJun expressing cells were inhibited in repair of cisplatin adducts (p = 0.55), whereas repair is readily detectable (p = 0.003) in parental cells. These observations indicate that the JNK/SAPK pathway is activated by cisplatin-induced DNA damage and that this response is required for DNA repair and viability following cisplatin treatment. Regulation of DNA repair following genotoxic stress may be a normal physiological role of the JNK/SAPK pathway.

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JNK/SAPK[1] is part of a kinase cascade that phosphorylates the transcription factor c-Jun at serine residues 63 and 73 (1–9). Phosphorylation of c-Jun at these sites greatly enhances the transactivation potential of the AP-1 binding sites (1–4) and AP-1 regulated genes *in vivo* (5, 11, 12), and there is evidence suggesting roles for c-Jun phosphorylation in cellular transformation (1, 2), inflammation (14), and apoptosis (15). The JNK/SAPK pathway is strongly stimulated in a dose-dependent manner by various DNA damaging treatments, including UV-C (5, 7–8), ionizing radiation (16), and alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (5), methylmethanesulfonate (MMS) (11), 1-β-D-arabinofuranosylcytosine (Ara-C) (17), and hydrogen peroxide (18). These observations suggest that the JNK/SAPK pathway may mediate a physiological response to DNA damage such as induction of one or more DNA repair enzymes. Here we provide evidence that the chemotherapeutic agent cisplatin, which damages DNA through the formation of bifunctional platinum adducts, but not transplatin, which does not damage DNA (19, 20), activates JNK/SAPK up to 10-fold in a dose-dependent manner. Furthermore, inhibition of this pathway in cells modified by expression of a nonphosphorylatable dominant negative mutant of c-Jun, dnJun, blocks DNA repair as judged by quantitative PCR and markedly decreases viability following treatment with cisplatin but not transplatin. Thus, JNK/SAPK is activated by cisplatin-induced DNA damage and is required for DNA repair and survival following cisplatin treatment.

EXPERIMENTAL PROCEDURES

Cells—Culture conditions and all cell lines and plasmids used here were developed using standard methods as described previously (22, 23). The expression of c-Jun and dnJun was quantitated using the methods (24) and antibodies previously characterized (24).

PCR—The PCR-stop assay (28) was used to quantitate cisplatin-DNA adduct formation and subsequent repair. The assay is based on the observation that the efficiency of amplification of cisplatin-treated DNA is inversely proportional to the degree of platination. Genomic DNA was isolated immediately or 6 h after treatment of cells for 1 h 15 min with varying amounts of cisplatin and amplified quantitatively using ^{32}P-end-labeled primers, giving rise to a 2.7-kb and a nested 0.15-kb fragment of the hyopxanthine phosphoribosyl transferase gene. The 5′ and 3′ primers were TGGGATTACACGTGTGAACCAACC and GATCCACAGTCTGCCTGAGTCACT, respectively, with a 5′ nested primer of CCTAGAAAGCACATGGAGAGCTAG. The 0.15-kb segment of genomic DNA sustains undetectable levels of DNA damage under our conditions and serves as an internal PCR control and the basis for normalization of the amount of amplification of the 2.7-kb fragment. The number of lesions/2.7-kb fragment (*i.e.* Fig. 4) is calculated as 1 − (cpm damaged DNA/cpm undamaged DNA) (8).

JNK Assay—JNK assays were carried out exactly as described previously (7).

Cytotoxicity—Viability (29) was assessed by the addition of cisplatin or transplatin for 1 h one day after seeding test cells into 96-well plates followed by a change of medium to fresh medium and determination of surviving cells 5 days later by addition of MTS for 1 h and determination of $A_{590\ nm}$ of the dissolved formazan product as described by the

[1] The abbreviations used are: JNK, c-Jun N-terminal kinase; AP-1, activator protein complex 1; cisplatin, *cis*-diaminodichloroplatinum; SAPK, stress-activated protein kinase; MTT, micro-tetrazolium (dye) test; transplatin, *trans*-diaminodichloroplatinum; UV-C, ultraviolet light C band, 254 nm maximum intensity for UV cross-linker 1800; dnJUN, dominant negative c-Jun; PCR, polymerase chain reaction; kb, kilobase pair; ATF, activation transcription factor; CREB, cAMP response element binding protein; MTS, (3-(4,5′-dimethylthiazol-2-yl)-5-(3-carboxymethoxylphenyl-2-(4-sulfophenyl)-2H-tetrazolium inner salt.

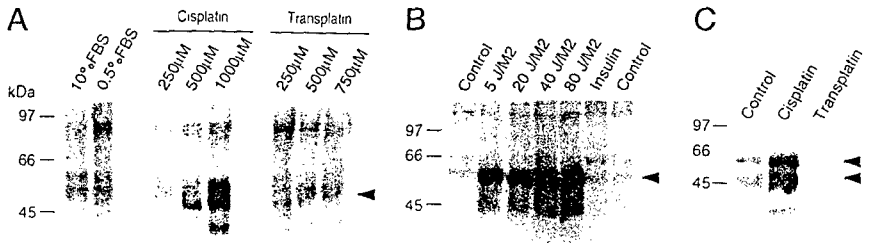

FIG. 1. **Cisplatin is a stereo-specific activator of JNK.** *A*, T98G human glioblastoma cells were stored overnight in serum-free medium, plated the next day, and treated by the indicated concentrations of cisplatin or transplatin for 1 h with a 1-h chase period followed by lysis and assay for JNK activity as described (7). Matching wells of cells were harvested and counted (Coulter counter) and used as the basis for sample loading. *FBS*, fetal bovine serum. *B*, positive control. T98G cells were exposed to the indicated doses of UV-C band (Stratalinker® UV cross-linker 1800) radiation and processed as described for *A*. *C*, JNK activity of human lung carcinoma M103 cells following treatment with 200 μM cisplatin or transplatin and processing as described for *A* with the addition of a 1-h chase prior to lysis.

manufacturer (Promega). All results were carried out in quadruplicate, and viability is expressed as the ratio of the amount of viable cells following cisplatin or transplatin treatment to that of the same cells without treatment.

RESULTS

Activation of JNK/SAPK by Cisplatin Requires DNA Adduct Formation—It is known that cisplatin but not transplatin forms covalent covalent cross-links between the N^7 position of adjacent guanine or adjacent adenine-guanine residues (19, 20). We find that the JNK activity of T98G cells is elevated in a dose-dependent manner up to 10-fold following a 1-h exposure to cisplatin but not to transplatin (Fig. 1*A*). As a positive control of the effects of a DNA-damaging agent, we examined the response of JNK of T98G cells to UV-C irradiation (Fig. 1*B*) and observed a similar dose-response relationship. Cisplatin-specific responses have been observed in other cell lines from tumor types that are commonly refractory to cisplatin treatment such as the human nonsmall cell lung carcinoma lines A549 (data not shown) and M103 (Fig. 1*C*). Moreover, 1 h after treatment with cisplatin, but not transplatin, JNK activity of T98G cells or lung carcinoma cells M103 remains elevated, suggesting that treatment with cisplatin leads to a prolonged response. These results indicate that only the DNA-damaging cisplatin isomer activates JNK activity.

Dominant Negative c-Jun Sensitizes Tumor Cells to Cisplatin but Not Transplatin—We developed clonal lines of human T98G glioblastoma cells, which stably express a dominant negative inhibitor (1, 2) of the JNK/SAPK pathway, dnJun. Expression of dnJun has no effect on either basal AP-1 activity (1, 2) or on the enzyme activity of JNK (data not shown) but does inhibit phosphorylation-dependent activation of transcription (1, 2, 10, 12). The effect of cisplatin treatment on the viability of representative clonal lines of the dnJun-expressing T98G cells is compared with that of an empty vector control line, T98GLHCX, in Fig. 2*A*. The viability of empty vector control T98G cells remains largely unaffected by treatment with increasing concentrations of cisplatin even at doses of ≥70 μM. Extended titrations revealed IC_{50} values of 147 and 154 μM for the parental cells and empty vector control cells, respectively (Table I). In contrast, the dnJun expressing cells exhibit an IC_{50} as low as 21 μM (Fig. 2*A*) or over 7-fold more sensitive to cisplatin than the control cells (Table I). Replicate experiments using additional clones that exhibit varying amounts of steady state dnJun indicate the sensitization to cisplatin is proportional ($r_{Pearson}$ = 0.98) to the amount of dnJun expressed (Fig. 2*B*). Transplatin has no discernible effect at concentrations where the viability following treatment with cisplatin is less than 25% (Fig. 3*B*) and in extended titrations no significant effect at 250 μM, indicating that the requirements for sensitization by dnJun depends upon the stereospecific DNA-binding properties of cisplatin, similar to the conditions for the activation of JNK.

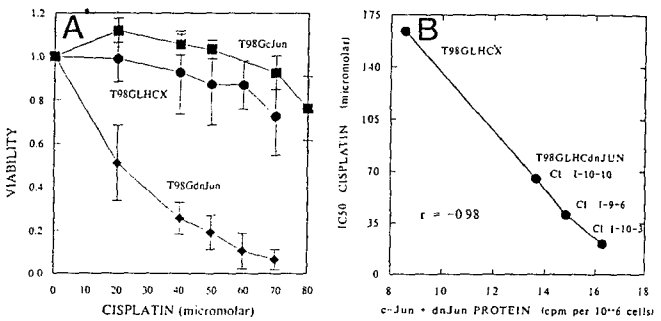

FIG. 2. **dnJun sensitizes T98G cells to cisplatin.** *A*, viability assay of empty vector control cells (●), wild-type c-Jun expressing cells (■), and clonal dnJun-expressing cells (◆). *B*, dose-response curve of the $IC_{50}^{cisplatin}$ of dnJun-expressing clones of T98G cells *versus* total immunoreactive c-Jun (*c-Jun* + *dnJun*). Total immunoreactive Jun (*c-Jun* + *dnJun*) was determined by sequential immunoprecipitation of ^{35}S-labeled cells using specific Jun B, Jun D, followed by pan-Jun antiserum as described previously (24). Values above 8.5 are taken as expression of dnJun.

Expression of wild-type c-Jun does not mimic dnJun-expressing cells (Fig. 2*A*). In fact, the viability of these cells when treated with cisplatin is somewhat *increased* relative to parental or empty vector control cells for all viability determinations in the range 20–60 μM cisplatin, suggesting that increased JNK substrate augmented viability following treatment with cisplatin (Fig. 2*A*). Thus, the sensitization to cisplatin observed for the dnJun-expressing cells appears to correlate with interference in the role of activated c-Jun.

Generality—We have tested the generality of the sensitizing properties of dnJun in PC3 prostate carcinoma cells modified to express dnJun under the control of an inducible truncated metallothionein promoter as described previously (21). Viability studies show that parental or empty vector control cells are largely insensitive to cisplatin at concentrations ≤60 μM (Fig. 3*A*, *circles*). Extended titrations revealed IC_{50} values of 109 and 156 μM for the parental and empty vector control cells, respectively (Table I). However, for PC3 cells that stably express pMTdnJun, the IC_{50} value is markedly reduced (Fig. 3*A*, *open diamonds*). Further, induction of maximum expression of dnJun by the addition of zinc acetate leads to greatly increased cytotoxicity with an IC_{50} of 16 μM (Fig. 3*B*) or 7.24–9.8-fold more sensitive to cisplatin than control cells (Table I). The addition of zinc acetate alone has no effect on the viability of parental or empty vector control cells (Fig. 3*A*, *filled circles*). Thus, the results observed following induction of expression of dnJun by a single clonal line confirm the results of Fig. 2*B* that sensitization to cisplatin is dependent upon the expression of dnJun.

As a further control, we examined PC3 prostate carcinoma cells modified to express a zinc-inducible c-Jun derivative, TAM-67, a well characterized transdominant negative inhibi-

TABLE I

Sensitization of human tumor lines to cisplatin-induced cytotoxicity

IC_{50} values were determined by direct titration of viability with cisplatin as described ("Experimental Procedures"). None of the cell lines examined here were made cisplatin-resistant prior to analysis.

Cell	Control[a]		dnJun-expressing IC_{50}	Cisplatin sensitization[b] $(IC_{50})^{Parent}/(IC_{50})^{dnJun}$
		IC_{50}		
		μM	μM	
T98G glioblastoma	Parental	140 ± 13	21 ± 3	7.0
	Empty vector pLHCX	154 ± 13		7.60
U87 glioblastoma	Parental	130 ± 53	50 ± 5	2.6
	Empty vector pLHCX	ND		
PC3 prostate carcinoma	Parental	109 ± 13	16 ± 2	7.2
	Empty vector pMT64AA	156 ± 18		9.2
MCF-7 breast carcinoma	Parental	145 ± 25	38 ± 2	3.8
	Empty vector pLHCX	101 ± 9		2.7

[a] In all cases parental and empty vector cells were analyzed in parallel and with equal concentrations of cisplatin and transplatin in the range 0–250 μM all in quadruplicate. Transplatin had no effect on viability of any cell. ND, not done.

[b] Sensitization is defined by the ratio of IC_{50} values for the parental or empty vector control cells to the IC_{50} value of the dnJun-expressing cells.

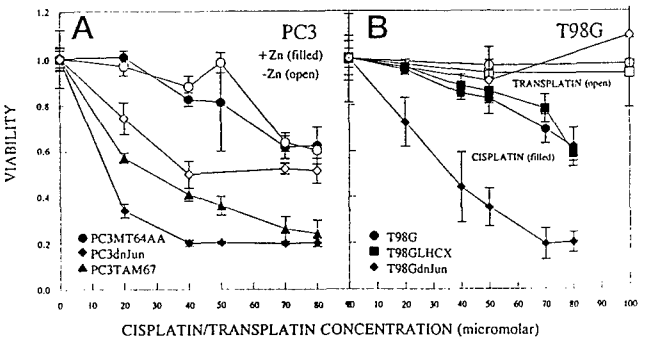

FIG. 3. **Sensitization of cells to cisplatin is general among cell types and stereospecific.** *A*, comparison of the viability of PC3 human prostate carcinoma cells modified to express pLHCX and the empty vector pMT64AA in the presence (■) or absence (●) of 25 mM zinc acetate to clonal PC3 cells the containing pLHCX and either the inducible vector pMTdnJun (♦) or pMTTAM-67 (▲) both in the presence of 25 mM zinc acetate. *B*, the viability of parental and modified T98G cells in the presence of cisplatin (*solid symbols*) or transplatin (*unfilled symbols*).

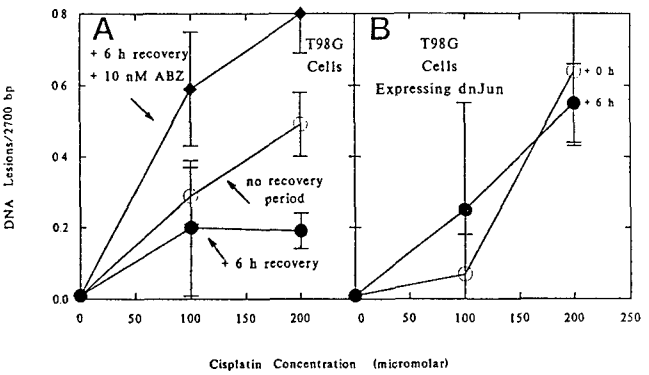

FIG. 4. **Expression of the transdominant inhibitor, dnJun, blocks cisplatin-induced DNA repair.** PCR results for the 2.7-kb segment of the hyopxanthine phosphoribosyl transferase gene was determined for 0, 100, or 200 μM cisplatin for 1 h as described ("Experimental Procedures") and expressed as 1 − (normalized efficiency of PCR amplification), a measure of cisplatin-induced lesions (10). *A*, PCR results for T98G parental cells either immediately or 6 h after treatment with cisplatin. The results are the averages of three assays for each of two independent preparations of DNA for the three concentrations of cisplatin. *B*, comparison of T98G cells and dnJun-expressing cells 6 h after treatment with cisplatin. The results are the averages of three assays. *ABZ*, 2-aminobenzidine.

tor of AP-1 owing to a deletion of residues 2–122 (21). As with dnJun, induction of TAM-67 in PC3 cells strongly enhances their sensitivity to cisplatin (Fig. 3*A*). We have determined that these TAM-67 and dnJun are expressed in approximately equal amounts, suggesting that the comparable degree of sensitization for TAM-67 and dnJun (Fig. 3*A*) is accounted for by interference in the role of phosphorylation-related function of c-Jun.

Similar results have been observed with an additional human glioblastoma line, U87, and an additional epithelial tumor line, MCF-7 (Table I). Clonal dnJun-expressing lines of these cells exhibit 2.6- and 3.8-fold decreased IC_{50} values, respectively (Table I). Thus, the sum of results indicate that the JNK/SAPK pathway may have a general role in mediating a functional response to DNA-cisplatin adduct formation. Inhibition of this response sensitizes cells to the cell-killing properties of cisplatin.

Cisplatin Activates and dnJun Inhibits DNA Repair—We assessed the extent of genomic DNA damage and repair following cisplatin treatment using a modified PCR assay (25). For this assay, it has been shown that the degree of inhibition of PCR-catalyzed amplification of DNA purified from *cisplatin-treated* cells is a direct measure of the amount of DNA-cisplatin adduct formation as measured by atomic absorption (25). Thus, this assay provides a direct assessment of the extent of cisplatin-induced DNA damage.

DNA isolated from T98G cells immediately after treatment with 0, 100, or 200 μM cisplatin for 1 h exhibit increasing levels of DNA damage (Fig. 4*A*, *circles*). However, if a 6-h "recovery" period is introduced prior to the DNA purification, damage is markedly and significantly ($p = 0.003$) reduced (Fig. 4*A*, *filled circles*). As a positive control for the effects of inhibition of genomic DNA repair, an inhibitor of ADP-ribosylation, 2-aminobenzidine, was added at the time of treatment of the cells with cisplatin (Fig. 4*A*, *squares*). Following the 6-h recovery period, DNA damage remained unrepaired, and total DNA damage was substantially increased. Next, we compared the level of DNA damage for T98G cells and dnJun-expressing cells following treatment with cisplatin (Fig. 4*B*). For the dnJun-expressing T98G cells, *6 h after* cisplatin treatment DNA damage remains completely unrepaired for cells treated at either 100 or 200 μM cisplatin ($p > 0.53$). All the results summarized here (Fig. 4, *A* and *B*) are the averages of three independent assays, which confirms the reliability of this observation. The sum of results, therefore, strongly indicates that expression of dnJun by T98G cells largely abolishes DNA repair following exposure of the cells to cisplatin.

DISCUSSION

These studies show that the JNK/SAPK pathway is activated by cisplatin-induced DNA damage and is required for DNA repair and viability following cisplatin treatment. T98G glioblastoma cells modified to express a nonphosphorylatable dom-

inant negative inhibitor of c-Jun, dnJun, fail to repair cisplatin adducts and are sensitized to the cytotoxic effects of cisplatin under conditions that have little or no effect on parental and control lines. In contrast, cell lines modified to overexpress wild-type c-Jun are resistant to cisplatin, an observation that rules out that possibility that the sensitization effect of dnJun is mediated by one or more of the domains it shares with wild-type c-Jun. Moreover, sensitization to cisplatin by dnJun is exhibited by several cell lines of varying origins. Sensitization to cisplatin is also observed in PC-3 prostate carcinoma cells modified to express TAM-67, a known dominant negative inhibitor of AP-1 (21). Because the degree of protein expression and sensitization is similar for TAM-67 and for dnJun, we conclude that most of the sensitization effects we observe are accounted for by inhibition of the phosphorylation-related functions of Jun.

Two major types of DNA regulatory elements that respond to the phosphorylation state of c-Jun include classic AP-1 sites and ATF/CREB sites. Classic AP-1 sites consisting of a 7-base pair consensus motif, T(G/T)A(C/G)TCA, bind to AP-1 complexes consisting of heterodimers of members of the Fos and Jun families and to Jun-Jun homodimers (9, 13–15, 21, 22, 26). ATF/CREB sites consisting of an 8-base pair consensus motif, T(G/A)CGTCA, bind to c-Jun/ATF2 heterodimers. Indeed, because JNK phosphorylates ATF2 as well as c-Jun and promotes complex formation and binding to ATF/CREB sites, these sites are likely to be major targets of JNK-mediated regulation (9, 12, 14, 15). Several enzymes known to be involved in repair of DNA-cisplatin adducts and implicated in cisplatin resistance (20) contain ATF/CREB sites in their promoters including DNA polymerase β (27, 28), topoisomerase I (30, 31), and proliferating cell nuclear antigen, an accessory protein of DNA polymerase delta (32, 33). Moreover, transcription of these genes is known to be activated through the ATF/CREB sites upon stimulation by genotoxic agents (27–33). Thus, the inhibition of induction of any or all of these activities could account for the inhibitory effects of dnJun on DNA repair and the resultant increase in cisplatin sensitivity. In view of the common regulatory mechanism involving ATF/CREB sites, a concerted induction of genes with a related function, DNA repair, is suggested. The sum of results indicate, therefore, that a potential physiological role for the strong activation of the JNK/SAPK pathway following DNA damage may be to mediate DNA repair by enhancing transaction of DNA repair enzymes.

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Addendum – During the review of this manuscript we became aware that activation of JNK/SAPK by cisplatin has been reported by Liu *et al.* (Liu, Z.-G., Baskaran, R., Lea-Chou, E. T., Wood, L. D., Chen, Y., Karin, M., and Wang J. Y. J. (1996) *Nature* **384,** 273–276).

REFERENCES

1. Smeal, T., Binétruy, B., Mercola, D. A., Birrer, M., and Karin, M. (1991) *Nature* **354,** 494–496
2. Smeal, T., Binétruy, B., Mercola, D. A., Bardwick-Grover, A., Heidecker, G., Rapp, U., and Karin, M. (1992) *Mol. Cell. Biol.* **12,** 3507–3513
3. Binétruy, B., Smeal, T., and Karin, M. (1991) *Nature* **351,** 122–127
4. Pulverer, B., Kyriakis, J. M., Avruch, J. Nikolakakie, E., and Woodgett, J., (1991) *Nature* **353,** 670–674
5. Dérijard, B., Hibi. M., Wu I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R., J. (1994) *Cell* **76,** 1025–1037
6. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) *Nature* **369,** 156–160
7. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes & Dev.* **7,** 2135–2148
8. Adler, V., Fuchs, S., Y. Kim, J., Kraft, A., King, M., P. Pelling, J., and Ronai, Z. (1995) *Cell Growth & Differ.* **6,** 1437–1446
9. Sluss, H., Barrett, T., Dérijard, B., and Davis, R. J. (1994) *Mol. Cell. Biol.* **14,** 8376–8384
10. van Dam, H., Wilhelm, D., Herr, I., Steffen, A., Herrlich, P., and Angel, P. (1995) *EMBO J.* **14,** 1798–1811
11. Adler, V., Franklin, C., and Kraft, A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5341–5345
12. van Dam, H., Duyndam, M., Rottier, R., Bosch, A., de Vries-Smits, L., Herrlich, P. Zantema, A., Angel, P., and van der Eb, A. J. (1993) *EMBO J.* **12,** 479–87
13. Whitmarsh, A., Shore, P., Sharrocks, A., and Davis, R. J. (1995) *Science* **269,** 403–407
14. Davis, R. (1994) *Trends Biochem. Sci.* **19,** 470–473
15. Xia, Z., Dickens, M., Raingeaud, J., Davis. R., and Greenberg, M. (1995) *Science* **270,** 1326–1331
16. Macgregor, P. F., Abate, C., and Curran, T. (1990) *Oncogene* **5,** 451–458
17. Kharbanda, S., Pandey, P., Ren, R., Mayer, B., Zon, L., and Kufe, D. (1995) *J. Biol. Chem.* **270,** 30278–30281
18. Yu, R., Jiao, J., Duh, J., Tan, T. H., and Kong, A. (1996) *Cancer Res.* **56,** 2954–2959
19. Sherman, S. E., Gibson, D., Wang, A. H., and Lippard, S. J. (1985) *Science* **230,** 412–417
20. Zamble, D. B., and Lippard, S. J. (1995) *Trends Biochem. Sci.* **20,** 435–439
21. Brown, P. H., Chen, T. K., and Birrer, M. J. (1994) *Oncogene* **9,** 791–799
22. Mercola, D. A., Carpenter, P., Grover-Bardwick, A., and Mercola, M. (1992) *Oncogene* **7,** 1793–1803
23. Potapova, O., H. Fakhrai, S. Baird and Mercola, D. (1996) *Cancer Res.* **56,** 280–286
24. Grover-Bardwick, A., Adamson, E., and Mercola, D. (1994) *Carcinogenesis* **15,** 1667–1674
25. Jennerwein, M. M., and Eastman, A. (1991) *Nucleic Acids Res.* **19,** 6209–6214
26. Karin, M., and Smeal (1992) *Trends Biochem. Sci.* **17,** 418–422
27. Kedar, P. S. Widen, S. G., Englander, E. W., Fornace, A. J. Jr., and Wilson, S. H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3729–3733
28. Horton, J., Srivastava, D. Zmudzka, B., and Wilson, S. H. (1995) *Nucleic Acids Res.* **23,** 3810–3815
29. Gjerset, R., Turla, S., Sobol, R., Scalise, J., Mercola, D., Collins, H., and Isabella, P. (1995) *Mol. Carcinogen.* **14,** 275–285
30. Heiland, S,. Knippers, R., and Kunze, N. (1993) *Eur. J. Biochem.* **217,** 813–822
31. Baumgartner, B., Heiland, S., Kunze, N., Richter. A., and Knippers, R. (1994) *Biochim. Biophys. Acta* **1218,** 123–127
32. Feuerstein, N., Huang, D., and Prystowsky, M. B. (1995) *J. Biol. Chem.* **270,** 9454–9458
33. Huang, D., Shipman-Appasamy, P. M., Orten, D., J., Hinrichs, S. H., and Prystowsky, M. B. (1994) *Mol. Cell. Biol.* **14,** 4233–4243

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